ATM prevents DSB formation by coordinating SSB repair and cell cycle progression

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DNA single-strand breaks (SSBs) arise as a consequence of spontaneous DNA instability and are also formed as DNA repair intermediates. Their repair is critical because they otherwise terminate gene transcription and generate toxic DNA double-strand breaks (DSBs) on replication. To prevent the formation of DSBs, SSB repair must be completed before DNA replication. To accomplish this, cells should be able to detect unrepaired SSBs, and then delay cell cycle progression to allow more time for repair; however, to date there is no evidence supporting the coordination of SSB repair and replication in human cells. Here we report that ataxia-telangiectasia mutated kinase (ATM) plays a major role in restricting the replication of SSB-containing DNA and thus prevents DSB formation. We show that ATM is activated by SSBs and coordinates their repair with DNA replication. SSB-mediated ATM activation is followed by a G₁ cell cycle delay that allows more time for repair and thus prevents the replication of damaged DNA and DSB accrual. These findings establish an unanticipated role for ATM in the signaling of DNA SSBs and provide important insight into the molecular defects leading to genetic instability in patients with ataxia-telangiectasia.

DNA damage | DNA repair | DNA single-strand breaks | ATM activation | genome stability

It is estimated that a human cell repairs 10,000–20,000 DNA lesions each day. The majority of these lesions arise from the intrinsic chemical instability of DNA, resulting in DNA single-strand breaks (SSBs), hydrolytic loss of DNA bases, base oxidations, nonenzymatic methylations, and other chemical alterations (1). Among these, SSBs are one of the most dangerous spontaneous lesions; if left unrepaired, they can terminate gene transcription and generate toxic DNA double-strand breaks (DSBs) during DNA replication (2, 3). The efficient repair of SSBs is important for the maintenance of genome stability, and SSB repair deficiency has been implicated in many human diseases, including neurodegeneration and cancer (4). To ensure that SSBs are repaired before DNA replication, cells need to detect unrepaired SSBs, report DNA damage to the checkpoint proteins, and delay cell cycle progression to allow more time for repair; however, to date there is no evidence supporting the existence of such a signaling pathway in human cells.

Ataxia-telangiectasia mutated kinase (ATM) plays a fundamental role in regulating the cellular response to stress by controlling cell cycle checkpoints. ATM-dependent cellular responses are activated by DSBs (5–7) and require the MRN complex, consisting of Mre11, Rad50, and Nbs1 proteins, and other cofactors, including Tip60 acetyltransferase and PP2A protein phosphatase (reviewed in ref. 8). Recently, it has become evident that ATM is also activated by a number of DNA-damaging agents that cause base oxidation (9, 10) and alkylation (11, 12). Alkylated and oxidized DNA bases are removed mainly by the base excision repair (BER) pathway, which generates SSBs as intermediate products. The process of BER is initiated by a specific DNA glycosylase that excises a damaged base to generate an abasic site (AP site) that is cleaved by AP-endonuclease. The resultant SSB

contains a 3'-hydroxyl terminus and a sugar phosphate residue at the 5'-terminus, and is further processed by DNA polymerase β and finally sealed by the XRCC1-DNA ligase III α complex (13). However, because the ligation of SSBs is slower than the generation of incised DNA, this leads to a transitional accumulation of SSBs. Therefore, we suggested that ATM might be activated by unrepaired SSBs, thus activating the DNA damage response to delay DNA replication and allow more time for SSB repair.

Here we report the first, to our knowledge, experimental evidence showing that unrepaired SSBs induced by both spontaneous DNA damage and exogenous agents activate ATM. Moreover, we demonstrate that SSB-dependent activation of ATM leads to a G₁ cell cycle delay that provides additional time for DNA repair before replication and prevents formation of DSBs. Thus, we find that the signaling of DNA SSBs by ATM plays an important role in preventing the replication of damaged DNA and is a key event required for the maintenance of genome integrity.

Results

Deficiency in SSB Repair Leads to ATM Activation. In response to DNA damage, ATM undergoes autophosphorylation (14), thereby activating the DNA damage response (reviewed in refs. 5–7). The phenomenon of ATM activation by DSBs is well established (7), but the question of whether accumulated DNA base lesions and SSBs can activate ATM remains unanswered. ATM-deficient cells are sensitive to hydrogen peroxide and alkylating agents, both of which produce DNA lesions that are repaired by BER (9–12). It was previously reported (15) and subsequently confirmed by us (Fig. 1 *A* and *B*) that treatment of cells with hydrogen peroxide or methyl methanesulfonate

Significance

DNA is chemically unstable; therefore, human cells have to repair a great number and variety of DNA lesions each day. DNA single-strand breaks (SSBs) represent one of the most dangerous lesions because, if unrepaired, they lead to genetic instability and are implicated in many human diseases. Here we report a novel mechanism that detects SSBs and controls the timing of SSB repair, thereby preventing the duplication of damaged DNA and the accumulation of mutations. Our findings shed important light on the molecular defects associated with the human disease ataxia-telangiectasia, which is caused by mutations in the *atm* gene.

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Fig. 1. DNA SSBs activate ATM. (A and B) TIG-1 human primary fibroblasts were treated with 150 μ M hydrogen peroxide (A) or 1.5 mM MMS (B) for the indicated times. (C) TIG-1 cells were treated with Lipofectamine RNAiMAX transfection reagent in the absence or presence of 200 pmol MPG for 60 h and then either left untreated or treated with 1 mM MMS for 60 min. (D and E) TIG-1 cells were treated with Lipofectamine RNAiMAX reagent in the absence (control) or presence of siRNA (200 pmol) against XRCC1 for 60 h (D) or the indicated time points (E). In A–E, cells were pelleted by centrifugation, whole-cell extracts were prepared, and proteins were separated by 4–16% (A–D) or 3–8% (E) SDS/PAGE and analyzed with the antibodies indicated. pATM, ATM phosphorylated at Ser1981; ATM, total ATM; PAR, poly(ADP ribose) polymer. Panels show representative data from one of five independent experiments. The pATM signal is normalized (pATM_{norm}) to ATM and vinculin (3–8% SDS/PAGE) or actin (4–16% SDS/PAGE); p53 and p21 signals are normalized to actin.

(MMS) activates ATM autophosphorylation; therefore, we hypothesized that ATM is activated by SSBs arising during the repair of oxidative or alkylating DNA lesions.

To explore the links between SSB formation and ATM activation, we treated TIG-1 primary human fibroblasts with MMS (Fig. 1C). This resulted in activation of DNA repair and accumulation of SSBs, as detected by the synthesis of poly(ADP ribose) polymer (PAR), which is produced by poly(ADP ribose) polymerase 1 in response to SSB formation (16, 17). We found that PAR accumulation was accompanied by ATM activation, as shown by Ser1981 autophosphorylation (Fig. 1C, lane 2, and Fig. S1 A and B); however, when SSB formation was blocked by knocking down the expression of *N*-methylpurine-DNA glycosylase (MPG), which initiates BER via excision of the methylated DNA bases produced by MMS (18), both PAR formation and ATM activation were ablated (Fig. 1C, compare lanes 2 and 4). These results were confirmed using three different siRNA sequences against MPG to reduce the possibility of off-target effects (Fig. 1C and Fig. S24). Therefore, we conclude that excessive production of SSBs during the repair process, rather than the DNA base damage per se, activates ATM.

To validate the biological significance of these findings, we next demonstrated that ATM is directly activated by SSBs generated during the repair of endogenous DNA lesions. We found that down-regulation (70–80% reduction at the protein level) of the BER scaffold protein XRCC1 (X-ray repair cross-complementing protein 1), which is involved in the repair of endogenous SSBs (19), increased the number of SSBs (as detected by an increase in PAR synthesis) and activated ATM phosphorylation at Ser1981 (Fig. 1D). ATM activation by SSBs was further confirmed by increased expression of downstream DNA damage-response genes such as p53 and p21 (Fig. 1D). Importantly, although significant down-regulation of XRCC1 was already observed at 24 h after siRNA application (Fig. 1E, lane 2), ATM activation gradually developed over time following the accumulation of unrepaired SSBs, as detected by

PAR synthesis (Fig. 1E). Activation of ATM by endogenous lesions was further confirmed in HeLa cells and in TIG-1 cells using three different siRNA sequences against XRCC1 (Fig. S2 B and C).

ATM Activation Following SSB Accumulation Is Not Associated with DSB Formation. Taking into account our data, together with the fact that both MMS treatment and XRCC1 knockdown induce SSBs (19, 20), we suggested that SSBs trigger ATM activation. It was important to exclude the possibility that ATM was activated by DSBs generated during the replication of SSB-containing DNA, however; thus, we investigated the nature of the DNA strand breaks arising from XRCC1 knockdown. First, we found no detectable changes in histone H2AX phosphorylation, a hallmark of IR-induced DSB formation, on Western blot analysis (Fig. 2A), suggesting formation of few, if any, DSBs. Second, to provide further evidence for the absence of DSBs after XRCC1 knockdown, we carried out a series of Comet assays. Using an alkaline Comet assay, which detects both SSBs and DSBs, we observed an increase in the number of DNA strand breaks after XRCC1 loss. The level of strand breaks was close to that generated by the treatment of cells with 0.5 mM MMS for 30 min (Fig. 2B and C, Upper). However, using a neutral Comet assay that detects only DSBs (21) we observed no induction of strand breaks in XRCC1-depleted cells, although DSBs were clearly present in cells irradiated with 2 Gy (Fig. 2B and C, Lower). Taken together, these results indicate that all strand breaks observed in the alkaline Comet assay following XRCC1 knockdown are SSBs.

To eliminate any concerns that ATM activation was due to the presence of DSBs at levels below that detectable by Comet assays and Western blot analyses, we measured ATM activation in an MMS-treated quiescent cell culture of TIG-1 human fibroblasts (Fig. 3A, quiescent) with the same cells that were released to cycle for 8 h before MMS treatment (Fig. 3A,

Fig. 2. ATM activation following XRCC1 knockdown is not associated with DSBs. TIG-1 cells were treated with Lipofectamine RNAiMAX reagent in the absence (control) or presence of XRCC1 siRNA (200 pmol) for 60 h. (A) Cells were pelleted by centrifugation, whole-cell extracts were prepared, and proteins were separated by 4–16% SDS/PAGE and analyzed with the antibodies indicated. TIG-1 cells irradiated at a dose of 4 Gy served as a positive control for staining with γ H2AX (histone H2AX phosphorylated at Ser139) antibody. (B) Cells were analyzed by alkaline (Upper) and neutral (Lower) Comet assays. Cells treated with 0.5 mM MMS for 30 min or irradiated at a dose of 2 Gy served as positive controls for the alkaline and neutral Comet assays, respectively. The mean % tail DNA values with SDs from three independent experiments are shown. Statistically significant results comparing control and XRCC1 siRNA-treated cells or positive controls are represented by P values, as analyzed by Mann–Whitney U test. NS, not significant. (C) Representative Comet assay images from the experiments shown in B.

released), and also with an MMS-treated cycling population of cells (Fig. 3A, cycling). The “released” cells, which still remained in G_0/G_1 phase after 8 h (Fig. S34 and Table S1), provided an internal control to ensure that noncycling cells retained the ability to promote ATM signaling.

We observed ATM activation in all three cultures (Fig. 3A), indicating that it occurred in the G_0/G_1 phase of the cell cycle (i.e., before DNA replication), supporting the notion that SSBs activate ATM. We found greater ATM activation in the “quiescent” cells than in the “cycling” cells, possibly related to the stress caused by contact inhibition, as also indicated by the finding of comparable levels of ATM activation in the released cells and the cycling cells.

Finally, to exclude the role of DNA replication in ATM activation by SSBs, we investigated ATM activation in quiescent cells following XRCC1 knockdown using three different siRNA sequences (Fig. 3B–D and Fig. S3B and C). To demonstrate that these cells did not undergo DNA replication, we labeled them with EdU at the time of XRCC1 knockdown for the duration of the experiment (Fig. S3B). We then analyzed ATM activation and formation of pATM foci in the EdU-negative population of SSB-containing G_0/G_1 cells that did not enter S phase. We observed an increase in ATM autophosphorylation (Fig. 3B, Western blot) and found greater numbers of phosphorylated ATM foci in the XRCC1-depleted cells compared with controls (Fig. 3C and D). These data confirm that ATM

phosphorylation is induced by SSBs that accumulate in response to XRCC1 depletion, and demonstrate the absence of replication-associated DSBs.

ATM Activation by SSBs Promotes S-Phase Entry Delay. Although XRCC1 depletion reduces cellular DNA repair capacity, we did not find an increase in the level of apoptosis following XRCC1 knockdown. These results indicate that despite their BER deficiency, the cells were able to repair SSBs and prevent the deleterious consequences leading to apoptosis (Table S2). One possibility is that SSB-dependent ATM activation delays S-phase entry to provide additional time for SSB repair and thus prevent DSB formation. To determine whether this was the case, we depleted XRCC1 in TIG-1 cells in the presence of the ATM activity inhibitor, Ku55993 (Fig. 4A and B and Fig. S4). We found that the Ser1981 phosphorylation induced by XRCC1 knockdown was blocked up on inhibition, indicating that phosphorylation was a consequence of ATM self-phosphorylation rather than of its phosphorylation by another protein kinase (Fig. 4B, compare lanes 2 and 4; Fig. S4B; and Table S3). Cell cycle analyses showed that ATM inhibition resulted in partial ablation of the G_0/G_1 cell cycle delay induced by XRCC1 knockdown (Fig. 4A, compare lanes 2 and 4).

The requirement for SSB-dependent ATM activation for the cell cycle delay was further confirmed by observations showing that increased expression of the cyclin-dependent kinase inhibitor p21 after XRCC1 knockdown was reduced by ATM inhibition (Fig. 4B, compare lanes 2 and 4, and Fig. S4A). To confirm that G_0/G_1 arrest imposed by ATM in XRCC1-depleted cells arises from SSBs induced in G_1 phase, rather than from DNA strand breaks formed in cells that have entered S phase during XRCC1 depletion, we labeled replicating cells with EdU for the duration of the XRCC1 knockdown (Fig. S5 and Table S4). Cell cycle analyses showed a significant accumulation of EdU-negative cells in G_1 phase after XRCC1 knockdown compared with controls ($27.7 \pm 3.4\%$ in XRCC1 siRNA vs. $3.1 \pm 0.2\%$ in controls). Of note, the cell cycle delay was partially rescued by ATM inhibition ($12.7 \pm 2.8\%$).

Importantly, ATM inhibition released the XRCC1-depleted cells for replication (Fig. 4A, last column, Fig. S4B, and Table S3). The uncontrolled replication of the DNA containing SSBs triggered by ATM knockdown led to the conversion of endogenous SSBs into DSBs, as measured by the neutral Comet assay (Fig. 4C, last column) and shown by the increased formation of 53BP1 foci in S-phase cells (Fig. 4D and Fig. S6A). The formation of replication-associated DSBs on ATM inhibition was confirmed using MMS as a base-damaging agent (Fig. S6B and C). The ability of ATM to delay cell cycle progression in response to SSBs was found to be p53-dependent because it was ablated by p53 knockdown (Fig. 5A and B and Fig. S7).

We further confirmed the role of ATM in promoting cell cycle delay in response to SSBs using ATM-deficient cells (GM03487 and GM2052) derived from patients with ataxia-telangiectasia (A-T). We found that compared with normal cells (GM03489), A-T cells were unable to delay cell cycle progression in response to SSB accumulation after XRCC1 knockdown (Fig. 5C and D). These results show that ATM-proficient cells detect SSBs, delay S-phase entry, and orchestrate sufficient time to seal all SSBs before replication, thereby preventing DSB formation.

Discussion

The inherent chemical instability of DNA and the potential for endogenous DNA damage requires continual DNA repair to avoid genome instability. BER provides the primary repair pathway for many DNA lesions, including base modifications and SSBs (13). Although base lesions per se fail to activate ATM, we found that unrepaired SSBs activate the ATM signaling pathway to ensure their early detection and to coordinate timing of their repair with

Fig. 3. ATM activation by SSBs precedes DNA replication. (A) TIG-1 cells were grown to 60–70% confluency (cycling), grown to confluency and maintained in contact inhibition for 24 h (quiescent), or grown to confluency and maintained in contact inhibition for 24 h, followed by replating in fresh medium at a dilution of 1:5 for 8 h (quiescent population that was further released to cycle; released). Cells were either left untreated or treated with 1 mM MMS for 30 min. Cells were collected by centrifugation, and whole-cell extracts were prepared and analyzed by Western blot analysis. A portion of the cells were labeled with 10 μ M BrdU for 20 min, collected by trypsinization, and subjected to PI/BrdU FACS analysis (Fig. S3A and Table S1). (B) TIG-1 cells were synchronized by contact inhibition as above and analyzed by PI/BrdU FACS to confirm their cell cycle status. The cells were treated with Lipofectamine RNAiMAX reagent in the absence (control) or presence of XRCC1 siRNA (200 pmol) for a further 60 h. Cells were harvested and analyzed by Western blot analysis with the antibodies indicated. In A and B, the Western blot panels show representative data from one of five independent experiments. The pATM signal is normalized to ATM and actin (4–16% SDS/PAGE) or vinculin (3–8% SDS/PAGE). (C and D) Quiescent TIG-1 cells were depleted of XRCC1 using siRNA for 60 h (as in B) in the presence of 1 μ M EdU, and pATM foci were analyzed by immunofluorescence microscopy in EdU-negative (G_1 phase) cells. In C, the total numbers of pATM foci per cell (Upper) and the percentage of cells containing 0, 1–3, 4–6, 7–9, and ≥ 10 pATM foci per nucleus (Lower) are shown. Statistical data are presented as a mean $\pm$ SD of three independent experiments; *P* values were calculated with the Student two-tailed *t* test. In D, representative examples of pATM staining are shown. EdU staining and bright-field images are shown in Fig. S3B and C.

cell cycle progression. This is an unanticipated finding that highlights a fundamental role for ATM in regulating SSB repair capacity and restricting the replication of damaged DNA (Fig. 6).

Previously it was shown that ATM plays an important role in a signal transduction pathway that controls cell cycle arrest following DNA damage, and it was suggested that abnormalities in this pathway might contribute to tumor development (22). Moreover, it was demonstrated that ATM is activated by ionizing irradiation, and it was proposed that radiation-induced DSBs trigger ATM autophosphorylation (14). However, in addition to DSBs, ionizing irradiation results in a significant amount of DNA base lesions and SSBs (23) which also may contribute directly to ATM activation, as indicated in the present work. In contrast to SSB formation, spontaneous DSB formation is a rare event (4); thus, we propose that one function of ATM is to promote genome stability by controlling the timing of SSB repair to prevent DSB formation. If this assumption is correct, then ATM-proficient cells should be able to delay S-phase entry following the accumulation of SSBs, to allow more time for repair. In contrast, ATM-deficient cells are not able to detect unrepaired SSBs, and will produce DSBs as a result of the replication of SSB-containing DNA. In this work, we discovered that SSBs indeed activate ATM, leading to an S-phase entry delay that prevents DSB formation. We also found that ATM-deficient cells fail to orchestrate the timely repair of SSBs and accumulate postreplicative DSBs, thus confirming the major role of ATM in preventing DSB formation.

The question arises that if ATM plays such an important role in genome stability, then why should individuals with A-T survive and only later develop clinical features characteristic of A-T? The reason for this may be related to the robust SSB repair capacity of human cells. In normal cells, only a very small number of SSBs can escape repair before the start of replication; however, these unrepaired SSBs will be detected by the ATM-signaling pathway, leading to delayed replication and consequent repair. In contrast, A-T cells are unable to detect SSBs that escape repair and consequently will replicate their damaged DNA, thus gradually converting SSBs into potentially toxic DSBs. Repair of these postreplicative DSBs by nonhomologous DNA end-joining has the potential to lead to chromosome/chromatid deletions or genome aberrations that interfere with normal gene expression and spark the pathology associated with A-T.

Materials and Methods

Cell Lines. TIG-1 normal human primary fibroblasts (AG06173; Coriell Institute Cell Repository) were grown in DMEM supplemented with 15% FBS at 37 °C in a humidified atmosphere with 5% CO₂. GM02052 (A-T proband), GM03489, and GM03487 (mother and proband, respectively, from A-T family 516) fibroblast strains were obtained from the Coriell Institute Cell Repository and maintained in MEM supplemented with 15% FBS.

Western Blot Analysis. Whole-cell extracts were prepared as described previously (24). Proteins were separated by SDS/PAGE, and Western blots were prepared according to standard procedures (Novex). Primary antibodies were as follows: actin (ab6276; Abcam), ATM phosphorylated at Ser1981

Fig. 4. ATM promotes a cell cycle delay in response to unrepaired SSBs and prevents DSB formation. (A and B) TIG-1 primary fibroblasts were treated with Lipofectamine RNAiMAX reagent in the absence (control) or presence of either XRCC1 siRNA (200 pmol) or XRCC1 siRNA and ATM inhibitor KU-55933 (10 μ M) simultaneously for 60 h. In A, cells were pulse-labeled with 10 μ M BrdU, collected by trypsinization, and analyzed by PI/BrdU FACS. The data are presented as mean $\pm$ SD of five independent experiments, where *P* values represent statistically significant results for cells in G₁ phase, as analyzed by the Mann–Whitney *U* test. FACS graphs and average and SD numerical values are presented in Fig. S4B and Table S3. In B, whole-cell extracts were prepared and analyzed by Western blot analysis. Representative data from one of four independent experiments are shown. Signals of p21 are normalized to actin. Statistical data are provided in Fig. S4A. (C and D) TIG-1 cells were treated with Lipofectamine RNAiMAX reagent in the absence (control) or presence of either XRCC1 or XRCC1 and ATM siRNA (200 pmol each) simultaneously for 60 h and in the presence of 1 μ M EdU. In C, cells were analyzed by a neutral Comet assay. Data are presented as mean $\pm$ SD % DNA damage of five independent experiments. In D, EdU-positive (S phase) cells were analyzed by immunofluorescence microscopy for 53BP1 foci. The data are presented as the total number of 53BP1 foci per cell (Left) and the percentage of cells containing ≥ 4 or ≥ 10 foci per nucleus (Right). The distribution of 53BP1 foci is shown in Fig. S6A. Statistical data are presented as mean $\pm$ SD of five independent experiments. In C and D, *P* values represent statistically significant results comparing control vs. XRCC1 or XRCC1/ATM siRNA-treated cells, as analyzed using the Student two-tailed *t* test. NS, not significant.

(pATM; ab81292; Abcam), ATM (ab59541; Abcam), MPG (ab155082; Abcam), PAR (4335-AMC-050; Trevigen), XRCC1 (used for immunofluorescence assays; MS-1393-P; Thermo Scientific), p53 (sc-126; Santa Cruz Biotechnology), vinculin (NB100-65546; Novus Biologicals), p21 (2947; Cell Signaling), and histone H2AX phosphorylated at Ser139 (γ H2AX; 05-636; Millipore). Anti-rabbit XRCC1 (used for Western blot analyses) antibody was produced in-house. Secondary antibodies conjugated with Alexa Fluor 680 (Molecular Probes) and IRDye 800 (Rockland) fluorescent dyes were used. Detection and quantification was carried out using the Odyssey image analysis system (Li-Cor Biosciences).

DNA Damage and Inhibitor Treatments. Cells were treated with 1–1.5 mM MMS (Acros Organics) for 30 min, 150 μ M hydrogen peroxide (Sigma-Aldrich) for 15 min, or ionizing radiation using a GSR-D1 137Cs γ -irradiator

(RPS Services) at a dose rate of 1.8 Gy/min. ATM kinase inhibitor KU55933 (2-morpholin-4-yl-6-thianthren-1-yl-pyran-4-one; Calbiochem) was used at 10 μ M.

Gene Silencing. Transfections with siRNA were carried out using Lipofectamine RNAiMAX reagent (Life Technologies) according to the manufacturer's protocol. Cells were analyzed at 60 h after transfection. The following siRNA sequences were used (Eurogentec): MPG, 5'-AAGAAGCAGCGACCAGCUAGA-3' (25); MPG#2, 5'-ACCGCAGCAUCUAUUUCUCAA-3' (26); MPG#3, 5'-CCU-GUACGUGUACAUAUUUA-3' (26); XRCC1, 5'-AGGGAAGAGGAAGUUGGAU-3' (27); XRCC1#2, 5'-GGCAGACACUACCGAAAA-3' (28); XRCC1#3, 5'-GGAA-GAUUAAGACAUUGAG-3' (29); p53, 5'-AAGACUCCAGUGGUAUUCUAC-3' (30); ATM, 5'-ACAUAUCUACUCAAAGACAUU-3' (31); scrambled siContra#1,

Fig. 5. G₁-phase cell cycle delay in response to unrepaired SSBs is p53- and ATM-dependent. (A and B) TIG-1 primary fibroblasts were treated with Lipofectamine RNAiMAX reagent in the absence (control) or presence of XRCC1 or p53 siRNA alone or XRCC1 and p53 siRNA simultaneously (200 pmol each) for 60 h. (C and D) TIG-1 and GM03489 normal fibroblasts, as well as GM03487 and GM2052 fibroblasts derived from individuals with A-T, were depleted of XRCC1 using siRNA as above. GM03489 (mother) and GM03487 (proband) cells belong to the same family (AT family 516; Coriell Institute Cell Repository) and thus can be compared directly. In A and C, cells were pelleted by centrifugation, whole-cell extracts were prepared, and proteins were separated by 4–16% (A) or 3–8% (C) SDS/PAGE and analyzed with the antibodies indicated. Representative data from one of three independent experiments are shown. In C, pATM levels at high exposure (HE) and low exposure (LE) are shown. In B and D, cells were pulse-labeled with 10 μ M BrdU, collected by trypsinization, and analyzed by PI/BrdU FACS. FACS graphs relating to B are provided in Fig. S7. Statistical data are presented as mean $\pm$ SD of five independent experiments. *P* values represent statistically significant results comparing corresponding control samples vs. siRNA-treated cells in G₁ phase, as analyzed by the Mann–Whitney *U* test. NS, not significant.

Fig. 6. Model for the coordination of SSB repair and cell cycle progression by ATM. In ATM-proficient cells, unrepaired DNA SSBs activate ATM to promote a cell cycle delay that allows more time for SSB repair before replication. By doing so, DSB formation and the accumulation of mutations is prevented (left branch). In contrast, ATM-deficient cells are unable to detect unrepaired SSBs, and cell cycle progression leads to DSB formation and accumulation of mutations (right branch).

5'-AAACCCGAUAAUACGUUGCG-3' (27); and nontargeting siContr#2, 5'-AGG-UAGUGUAAUAGCCUUG-3'.

Immunofluorescence and Foci Detection. Slides were prepared as described previously (32). Primary pATM, XRCC1, and 53BP1 antibodies were as described above. Secondary antibodies were Alexa Fluor 488 (H+L) goat anti-rabbit and Alexa Fluor 594 (H+L) goat anti-mouse (Molecular Probes). For EdU labeling, cells were incubated in the presence of 1 μ M EdU for at least 1 h and then fluorescently labeled with Alexa Fluor 647 dye using the Click-iT

EdU Alexa Fluor 647 Imaging Kit (C10340; Life Technologies) according to the manufacturer's protocol. Imaging was carried out using a Zeiss LSM 780 confocal microscope and an 63 $\times$ oil objective. Slides for the detection of focus formation were prepared in duplicate, and 150 cells per slide were counted. The results were recorded as both foci per cell values and the percentage of cells with 4 or $\geq$ 10 foci.

Comet Assay. Control and siRNA-transfected cells were trypsinized and analyzed by alkaline and neutral Comet assays as described previously (21, 33).

Apoptosis and Cell Cycle Analysis. For apoptosis detection, cell cultures were collected and analyzed using an Annexin V kit (Abcam) as recommended by the manufacturer. For cell cycle analyses, cells were pulse-labeled with 10 μ M BrdU for 20 min, trypsinized, and fixed in ice-cold 70% ethanol for at least 30 min. After removal of the fixative solution by centrifugation, cells were incubated in 2 M HCl containing 0.1 mg/mL pepsin for 20 min, followed by washing in PBS and PBS supplemented with 2% FBS. Cells were incubated with primary antibody against BrdU (347580; BD Biosciences) and secondary antibody, and then further stained with 10 μ g/mL propidium iodide (Sigma-Aldrich) in PBS. Alternatively, samples were pulse-labeled with 10 μ M EdU for 1 h or with 0.5 μ M EdU for 60 h, fixed, fluorescently labeled with Alexa Fluor 647 dye using a Click-iT EdU Alexa Fluor 647 Flow Cytometry Assay Kit (C10634; Life Technologies), and stained with propidium iodide. Samples were run on a FACSCalibur flow cytometer (BD Biosciences), and the data were analyzed using FlowJo software.

Statistical Analysis. The Shapiro-Wilk test was used to examine datasets for a normal distribution. Statistical data are presented as mean $\pm$ SD of at least three independent biological experiments. *P* values were calculated with either the Student two-tailed *t* test for normally distributed datasets or the nonparametric Mann-Whitney two-tailed *U* test.

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